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TRAF6 Is a Critical Factor for Dendritic Cell Maturation and Development

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Summary

IL-1 receptor (IL-1R)/Toll-like receptor (TLR) family and TNF receptor (TNFR) superfamily members are critical for regulating multiple aspects of dendritic cell (DC) biology. Several signaling pathways associated with each family utilize the adapter molecule, TRAF6, but its role in DCs is unclear. By examining TRAF6-deficient mice and bone marrow (BM) chimeras reconstituted with TRAF6-deficient fetal liver cells, we show that proper DC maturation requires TRAF6. In response to either microbial components or CD40L, TRAF6-deficient DCs fail to upregulate surface expression of MHCII and B7.2, or produce inflammatory cytokines. Moreover, LPS-treated TRAF6-deficient DCs do not exhibit an enhanced capacity to stimulate naive T cells. Interestingly, a major population of splenic DCs, the $CD4^+CD8\alpha^-$ subset, is nearly absent in both TRAF6-deficient mice and BM chimeras. Together these results indicate that TRAF6 regulates the critical processes required for maturation, activation, and development of DCs, the primary cellular bridge between innate and adaptive immunity.

Introduction

Dendritic cells (DCs) specialize in capturing, processing, transporting, and presenting antigens to T cells (Banchereau et al., 2000; Banchereau and Steinman, 1998). DCs initiate primary T cell responses by presenting antigenic peptides on MHC molecules and providing costimulatory signals that are essential for optimal T cell responses. DCs are heterogeneous in phenotype, localization, and function. To date, the following three DC subsets have been described in the mouse spleen based on high surface expression of CD11c and differential expression of the conventional T cell markers CD4 and CD8α: $CD4^+$ $CD8\alpha^-$, $CD4^-$ $CD8\alpha^+$, and $CD4^-$ $CD8\alpha^-$ (Shortman and Liu, 2002; Vremec et al., 2000). $CD8\alpha^+$ DCs are localized mainly in the T cell areas, whereas the majority of $CD8\alpha^-$ DCs are found in the marginal zones but can be induced to migrate into the T cell areas upon activation (Banchereau et al., 2000). Although characterization of the different splenic DC subsets has been limited and remains unclear, it has been suggested that they determine the distinct nature of T cell responses (Th1 versus Th2; T cell activation versus tolerance) (Legge et al., 2002; Moser and Murphy, 2000).

Immature DCs in the periphery uptake antigens efficiently but express low levels of MHC and costimulatory molecules and thus possess a low level of T cell stimulatory capacity. Upon maturation DCs greatly augment their ability to stimulate naive T cells by dramatically upregulating surface expression of antigen/MHC complexes and various costimulatory molecules. DC maturation can be triggered by a response to microbial components (e.g., lipopolysaccharide [LPS], double-stranded RNA, or CpG-DNA) or inflammatory cytokines such as CD40L (Banchereau et al., 2000; Banchereau and Steinman, 1998). These stimuli also activate DCs to secrete cytokines including IL-12, which is critical for the differentiation of T cells into Th1 type effector cells (Trinchieri, 2003).

Recently, key regulatory factors for DC maturation and activation have begun to be identified. DCs can recognize microbial components using various pattern recognition receptors called Toll-like receptors (TLRs) (Janeway and Medzhitov, 2002). Different TLRs expressed on DCs can discriminate distinct pathogen-associated molecular patterns (PAMPs) and initiate signaling pathways to induce DC maturation and activation (Janeway and Medzhitov, 2002). For example, CpG-DNA utilizes TLR9 to induce DC maturation, cytokine production, and T cell stimulatory activity, while LPS stimulates DCs through TLR4 (Hemmi et al., 2000; Kaisho and Akira, 2001; Kaisho et al., 2001).

Upon recognition of their cognate ligands, TLRs recruit the cytoplasmic adaptor protein, MyD88, leading to the activation of various kinases (e.g., IKK, MAPK) and transcription factors (e.g., NF-κB) (Medzhitov et al., 1998; Muzio et al., 1998). DCs from MyD88-deficient mice fail to elaborate cytokines in response to CpG-DNA, LPS, and other microbial components, implicating MyD88 in the signaling pathways of most, if not all, TLRs.

However, further examination of DC maturation has revealed apparently distinct classes of TLRs, as CpG-DNA fails to upregulate surface expression of MHCII and other costimulatory molecules on MyD88-deficient DCs, whereas, surprisingly, LPS induces normal phenotypic maturation (Kaisho and Akira, 2001; Kaisho et al., 2001). This finding suggested the existence of a MyD88-independent signaling pathway(s) for TLR4-mediated induction of DC maturation. Therefore, two distinct intracellular signaling pathways appear to regulate DC maturation by different TLRs: one class (e.g., TLR9) is strictly dependent on MyD88 while the other (e.g., TLR4) can induce

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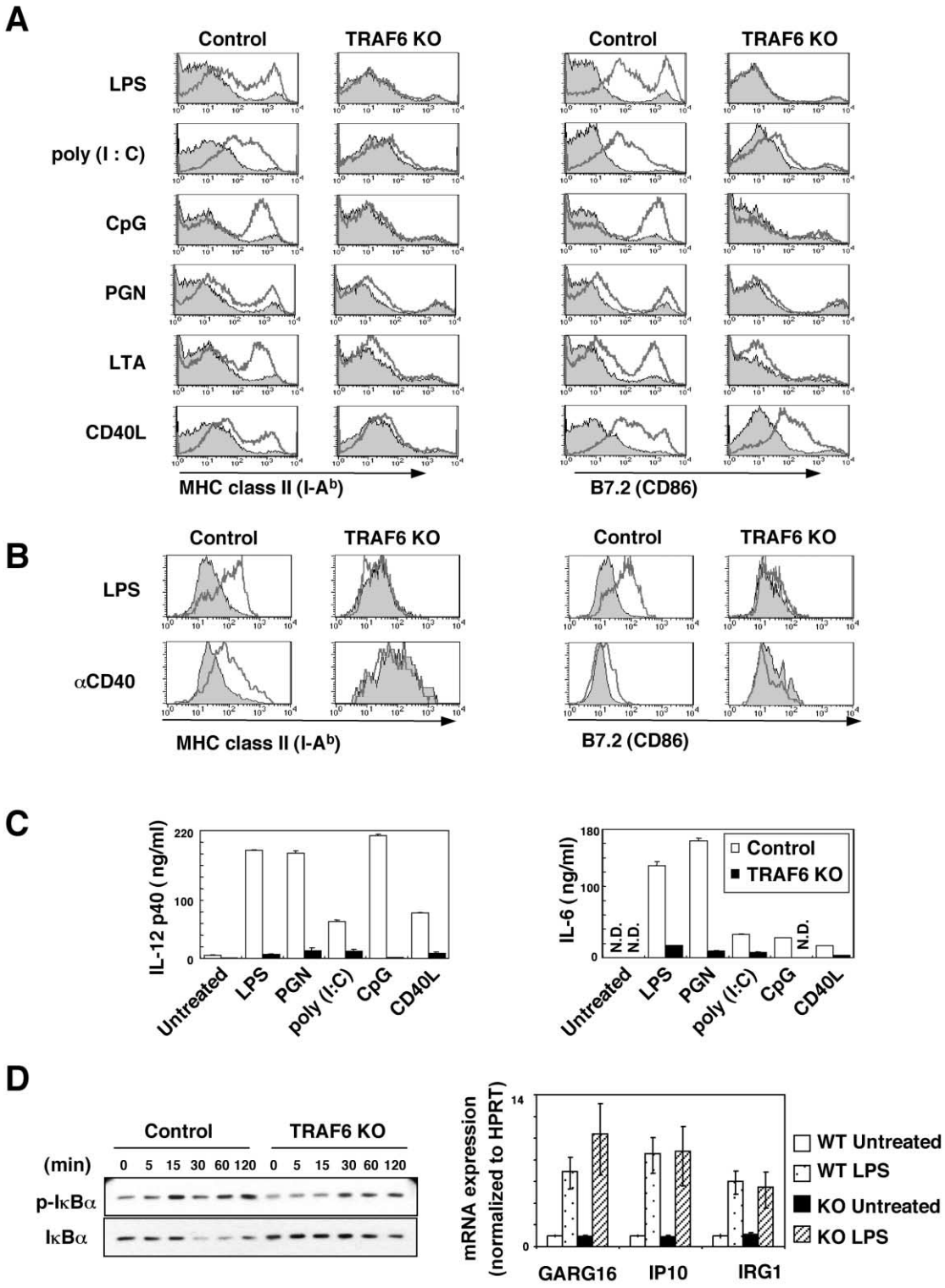

Figure 1. Phenotypic DC Maturation and Cytokine Production Are Impaired in TRAF6-Deficient DCs

(A) Phenotypic maturation of TRAF6$^{-/-}$ DCs is impaired in vitro. DCs were generated from the spleens of TRAF6$^{-/-}$ mice or control littermates in vitro. DCs were left untreated (filled histogram) or stimulated as indicated (open histogram), and surface I-A^{b} and B7.2 levels were analyzed by flow cytometry. Results are representative of at least three independent experiments.

(B) LPS- and CD40-induced DC maturation is impaired in TRAF6$^{-/-}$ mice in vivo. (Top) LPS or PBS was injected into TRAF6$^{-/-}$ and control mice. Six hours later, I-A^{b} and CD86 expression levels on CD11c^{high} DC populations were compared between PBS-injected (filled histogram) and LPS-injected (open histogram) mice. Results are typical of three independent experiments with similar results. (Bottom) Anti-CD40 Ab was injected i.p., and DCs were analyzed 27 hr later. I-A^{b} and CD86 expression levels on CD8α^{+} CD11c^{high} DC populations were compared

DC maturation in the absence of MyD88. Whether MyD88-dependent and -independent signaling pathways converge during TLR-induced DC maturation remains unknown.

Some of the transcription factors activated by TLR-MyD88 have also been implicated in the regulation of DC function (Ouaaz et al., 2002). It has been reported that p50/cRel double knockout DCs fail to produce IL-12 in responses to LPS and other microbial components. Therefore, it is likely that the MyD88-NF-κB (particularly the p50 and cRel subunits) signaling pathway is essential for inflammatory cytokine production by DCs stimulated through most, if not all, TLRs. However, p50/cRel double knockout DCs still phenotypically mature in response to LPS (Ouaaz et al., 2002), indicating that other factors may control TLR-mediated DC maturation.

Similar to TLRs, stimulation through the TNFR superfamily member CD40 leads to DC maturation and cytokine production. Recent studies have shown that CD40-induced cytokine production by DCs also depends on the activation of NF-κB subunits p50 and cRel (Ouaaz et al., 2002), suggesting that NF-κB activation may occur at the point of convergence for signaling pathways emanating from both CD40 and the TLR family, and resulting in inflammatory cytokine production by DCs. However, the existence of such a point of convergence for these distinct signaling pathways, as well as the specific identity of signaling mediators and transcription factors that regulate CD40-mediated DC maturation, remains uncertain.

TRAF6 is a member of the TRAF (TNF receptor-associated factor) family of proteins, which have been characterized as adaptor molecules that mediate signals induced by the TNFR superfamily (Arch et al., 1998; Chung et al., 2002). TRAF6 recognizes and binds to a peptide motif (PXEXXAr/Ac) in the cytoplasmic portion of CD40 and TRANCE receptor (TRANCE-R, also known as RANK) (Ye et al., 2002). Upon stimulation, interaction of TRAF6 with CD40 or TRANCE-R activates NF-κB transcription factors and mitogen-activated protein (MAP) kinases (Ishida et al., 1996; Kobayashi et al., 2001; Lomaga et al., 1999; Wong et al., 1998). A unique feature of TRAF6 is that, unlike other TRAFs, it also participates in IL-1R/TLR family signaling (Cao et al., 1996). In this pathway, TLR-MyD88 association activates IRAK, which in turn leads to TRAF6-mediated activation of the NF-κB and MAPK cascades (Medzhitov et al., 1998; Muzio et al., 1998). It seems, therefore, that TRAF6 functions at the central point where signals induced by the TLR and TNFR families converge.

To clarify the biological significance of TRAF6 in DCs, we have utilized TRAF6-deficient mice and bone marrow (BM) chimeras reconstituted with TRAF6-deficient fetal liver cells. We show here that the absence of TRAF6 causes impaired DC maturation in response to microbial components and CD40L. In addition, we show that TRAF6 is required for induction of cytokine production by DCs and for acquisition of enhanced T cell stimulatory capacity. Unexpectedly, we also found that TRAF6 deficiency results in a developmental defect in the generation of the splenic $CD4^+$ DC subset. On the basis of the results presented here, we propose a pivotal function of TRAF6 in the regulation of DC maturation and the development of DC subsets.

Results

Functional Disruption of TRAF6 in Mice

To disrupt the functional TRAF6 protein in vivo, we employed a knockin vector that enabled us to delete a floxed *TRAF6* exon 7 encoding the last zinc finger domain and the entire TRAF domain. TRAF6-deficient ($TRAF6^{-/-}$) mice appeared normal at birth, but became progressively runted, and typically died by 3 weeks of age. $TRAF6^{-/-}$ mice exhibited severe osteopetrosis, thymic atrophy, lymph node deficiency, and splenomegaly. Spleens from $TRAF6^{-/-}$ mice were found to be markedly disorganized, with a complete lack of normal T and B cell areas. Small clumps of lymphocytes were found scattered throughout splenic sections, but distinct periarteriolar or lymphoid collections were absent (data not shown). These observations are consistent with those of previously reported TRAF6-deficient mice lacking exon 3 or exon 4 (Lomaga et al., 1999; Naito et al., 1999).

Phenotypic Maturation and Cytokine Production Are Impaired in TRAF6-Deficient DCs

To determine the function of TRAF6 in DCs, we first examined phenotypic maturation of cultured DCs in vitro in response to various TLR ligands and CD40L. Since TRAF6 deficiency caused severe osteopetrosis, resulting in occlusion of the bone marrow cavity, we generated DCs from spleen cells of $TRAF6^{-/-}$ mice and age-matched control littermates. DCs were stimulated with LPS (for TLR4), poly (I:C) (for TLR3), CpG-DNA (for TLR9), peptidoglycan (PGN) (for TLR2), lipoteichoic acid (LTA) (for TLR4), or CD40L for 24 hr, followed by flow cytometric assessment of surface expression of MHC class II ($I\text{-}A^b$) and B7.2 (CD86). There was no difference in the surface expression levels of MHC class II or B7.2 between untreated immature $TRAF6^{-/-}$ DCs and control DCs.

Consistent with previous reports (Banchereau et al., 2000; Kaisho and Akira, 2001), MHC class II and B7.2 were strongly upregulated on wild-type DCs in response to all TLR stimuli tested and CD40L (Figure 1A). In contrast, upregulation of surface expression of MHC class II was significantly impaired in $TRAF6^{-/-}$ DCs in response to all stimuli tested (Figure 1A). Similarly, TLR ligands failed to upregulate B7.2 on $TRAF6^{-/-}$ DCs. CD40L, however, induced upregulation of B7.2 on

between PBS-injected (filled histogram) and anti-CD40-injected (open histogram) mice.

(C) Cytokine production is impaired in TRAF6-deficient DCs. DCs were generated from spleens of $TRAF6^{-/-}$ (filled column) or control littermates (open column). IL-12 p40 and IL-6 levels were measured by ELISA. Means ± SD of triplicate samples of one representative experiment out of three independent experiments are shown.

(D) DCs were stimulated with LPS for the indicated times. In the left panel, NF-κB activation was determined by Western analysis. In the right panel, the expression of IFN-inducible genes (GARG16, IP10, and IRG1) was examined by real-time PCR analyses.

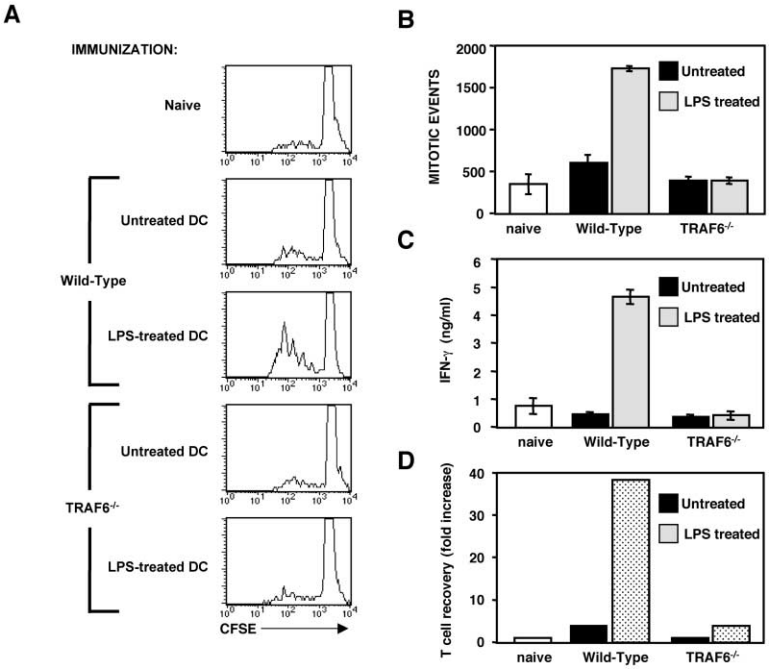

Figure 2. TRAF6 Is Required for Optimal Stimulation of T Cells by DCs

(A–C) TRAF6$^{-/-}$ DCs are defective in their capacity to prime allogeneic T cells in vivo. DCs were generated from spleens of TRAF6$^{-/-}$ mice or control littermates, and stimulated with LPS or left untreated. DCs were injected into Balb/c mice; then 7 days later, splenic T cells were isolated, labeled with CFSE, and restimulated for 3 days with T cell-depleted 129/Sv x C57BL/6 splenocytes. (A) CFSE profiles of restimulated CD3^{+} T cells from DC-primed Balb/c mice are shown. The data are representative of three independent experiments. (B) Mitotic events of restimulated T cells in (A) were determined as described in the Experimental Procedures. (C) IFN-γ production from restimulated T cells in (A) was measured by ELISA.

(D) TRAF6$^{-/-}$ BMDCs are defective in their capacity to prime antigen-specific T cells in vivo. OTII CD4^{+} T cells were adoptively transferred into recipient mice, followed 24 hr later by immunization with either PBS (open column), wild-type untreated (filled column), or LPS-treated (checked column) BMDCs, or TRAF6$^{-/-}$ untreated (filled column) or LPS-treated (checked column) BMDCs. The number of donor T cells recovered 4 days postimmunization relative to total recipient splenocytes and normalized to the PBS immunization condition is depicted. The data expressed is representative of the mean recovery obtained from duplicate mice.

TRAF6$^{-/-}$ DCs, although the expression levels were slightly reduced compared to those of control DCs. Similar results were obtained using cultured bone marrow DCs derived from TRAF6$^{-/-}$ fetal liver cell-reconstituted chimeric mice (data not shown). These results indicate that optimal DC maturation in vitro by TLR- or CD40-mediated stimulation is dependent on TRAF6. The TRAF6-independent mechanism by which CD40L induces expression of B7.2 remains unknown. It is possible that, in this context, the utilization by CD40 of additional TRAF proteins not used by the TLR family (e.g., TRAF2) may compensate for the lack of TRAF6.

We next examined in vivo phenotypic maturation of splenic DCs induced by LPS or CD40. Splenic CD11c^{high} cells from PBS-injected TRAF6$^{-/-}$ mice and normal littermates showed moderate but similar expression levels of MHC class II and B7.2 (Figure 1B). The expression levels of these surface molecules were strongly increased in control mice by injection of LPS or anti-CD40 antibody. However, neither LPS nor CD40 induced upregulation of surface molecules in TRAF6$^{-/-}$ mice. Together with the in vitro-cultured DC experiments, these results imply that TRAF6 is required for DC maturation in response to microbial components, such as LPS or the TNF superfamily member CD40L, both in vitro and in vivo.

We also measured IL-6 and IL-12 levels in the culture of DCs activated by TLR ligands or CD40L. Although both IL-6 and IL-12 levels were dramatically induced in the culture supernatant of wild-type DCs by all stimuli, cytokine production by TRAF6$^{-/-}$ DCs was significantly impaired (Figure 1C). These results indicate that CD40L and microbial components stimulate cytokine production from DCs in a TRAF6-dependent manner.

Despite defects in the maturation process of TRAF6$^{-/-}$ DCs, some biochemical responses to microbial products appear to remain intact. For example, LPS stimulation of TRAF6$^{-/-}$ DCs resulted in activation of NF-κB, though somewhat delayed and less robust in comparison to wild-type DCs (Figure 1D). In addition, LPS-induced expression of IFN-dependent genes by TRAF6$^{-/-}$ DCs is comparable to that of wild-type DCs (Figure 1D).

LPS-Induced T Cell Stimulatory Capacity of TRAF6-Deficient DCs Is Impaired

To determine the functional properties of TRAF6$^{-/-}$ DCs, we examined their capacity to induce T cell stimulation. First, the influence of LPS-treated or untreated DCs on in vivo allogeneic T cell priming was examined. Balb/c mice were injected with cultured DCs derived from TRAF6$^{-/-}$ or wild-type spleen (129/Sv x C57BL/6). Seven days later, splenic T cells from immunized mice were isolated and restimulated with T cell-depleted splenocytes from 129/Sv x C57BL/6 mice. The proliferative response to allogeneic antigens of T cells from mice primed with LPS-treated wild-type DCs was significantly enhanced compared to T cells from mice injected with untreated wild-type DCs (Figures 2A and 2B). In contrast, the proliferative response of T cells from mice primed with TRAF6$^{-/-}$ DCs was not augmented by LPS treatment, suggesting that TRAF6 is required for an LPS-induced increase in allostimulatory capacity of DCs.

We further investigated the allostimulatory capacity of TRAF6$^{-/-}$ DCs by assessing the production of Th1 (IFN-γ) and Th2 (IL-4 and IL-10) cytokines by T cells in an MLR. IFN-γ production by restimulated T cells primed in vivo with LPS-treated wild-type DCs was remarkably increased compared to that of T cells primed with untreated wild-type DCs (Figure 2C). In contrast, restimu-

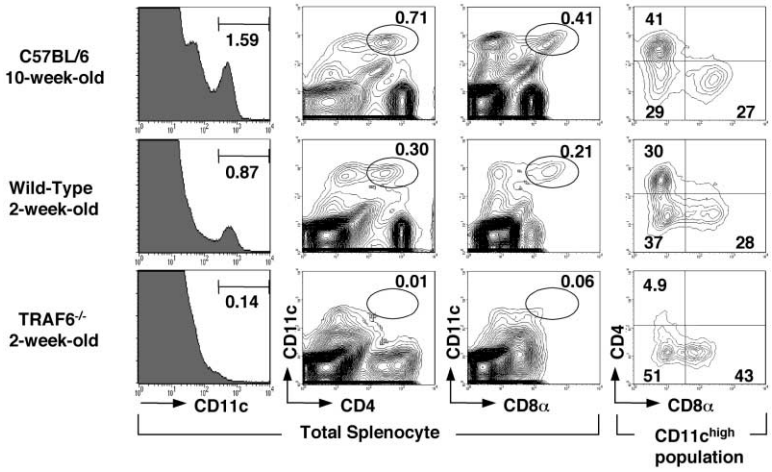

Figure 3. Splenic DC Development Is Severely Impaired in the TRAF6$^{-/-}$ Mouse

Splenocytes were stained for CD11c, CD4, and CD8α. Histograms of CD11c expression in total splenocytes are shown in the far left panels. Percentages of gated CD11c^{high} populations are shown. Profiles of CD11c versus CD4 and CD11c versus CD8α in total splenocytes are shown in the middle left and middle right panels, respectively. Percentages of the encircled CD4$^+$ DC and CD8α$^+$ DC populations are shown. Profiles of CD4 versus CD8α in CD11c^{high} DC populations are shown in the far right panels. The data are representative of at least three mice for each condition.

lated T cells primed with either LPS-treated or untreated TRAF6$^{-/-}$ DCs exhibited a low amount of IFN-γ production, which was consistent with a defective capacity for induction of allogeneic T cell proliferation. Thus, the LPS-induced Th1-like response evoked by TRAF6$^{-/-}$ DCs was largely abolished. However, IL-4 and IL-10 were below detectable levels in all conditions (data not shown), suggesting that the defect in IFN-γ production by restimulated T cells primed with LPS-treated TRAF6$^{-/-}$ DCs was not due to a skewing toward a Th2 response.

We have also examined the function of TRAF6$^{-/-}$ DCs in a T cell response to a model peptide antigen. For this, chicken ovalbumin (OVA)-specific OTII CD4$^+$ T cells were adoptively transferred into C57BL/6 mice, which were then immunized with OVA-loaded, LPS-treated, or untreated DCs. Four days postimmunization, recipient splenocytes and peripheral lymph node cells were assayed for the presence of donor OTII T cells. While very few OTII donor T cells were recovered from unimmunized control mice, the number increased by roughly 4-fold in mice immunized with untreated wild-type DCs and nearly 40-fold in mice immunized with LPS-treated wild-type DCs (Figure 2D). In contrast, the number of donor T cells recovered from mice immunized with untreated TRAF6$^{-/-}$ DCs was roughly equal to that of unimmunized mice, and the number found in mice injected with LPS-treated TRAF6$^{-/-}$ DCs was only roughly equivalent to the number found in mice immunized with untreated wild-type DCs. Additionally, CFSE dye dilution indicated that, whereas the vast majority of donor T cells recovered from mice injected with LPS-treated wild-type DCs had divided repeatedly, about half of those recovered from mice immunized with LPS-treated TRAF6$^{-/-}$ DCs failed to divide at all (data not shown). These data further suggest an inability of DCs to induce or potentiate either or both proliferation and survival of antigen-specific T cells in the absence of TRAF6-mediated signaling. Taken together, these results showed that, similar to an allogeneic response, TRAF6$^{-/-}$ DCs fail to induce peptide antigen-specific T cell responses.

Splenic DC Development Is Severely Impaired in the TRAF6$^{-/-}$ Mouse

TRAF6 is known to activate NF-κB family transcription factors, some members of which have been implicated in regulation of DC development (Ouaaz et al., 2002; Wu et al., 1998). Therefore, we examined whether TRAF6 regulates splenic DC development in vivo. As most TRAF6$^{-/-}$ mice die by 2.5–3 weeks of age, spleen cells from 2-week-old TRAF6$^{-/-}$ mice and age-matched littermates were used in these experiments. We have also included 10-week-old C57BL/6 spleens as a control for normal, adult mice.

Ten-week-old C57BL/6 mice exhibited a distinct population of CD11c^{high} cells (1.59 ± 0.34%) (Figure 3 and Table 1). Analysis of different DC subsets determined by surface expression of CD4 and CD8α on CD11c^{high} cells showed the CD4$^+$8α$^-$ population (CD4$^+$ DCs) to be the predominant DC subset (41.3 ± 2.5%) in control mice (Figure 3, right panel), a finding consistent with previous reports (Shortman and Liu, 2002). Although 2-week-old wild-type mice (129/Sv x C57BL/6) showed a lower frequency of CD11c^{high} cells (0.87 ± 0.19%) than 10-week-old control mice, a distinct CD11c^{high} population was still evident. However, unlike 10-week-old mice, all three subsets of DCs were present in roughly equal numbers (Figure 3 and Table 1). The apparent increase in the percentage of CD4$^+$ DCs with age does not seem to result from differences in genetic backgrounds as 4- to 10-week-old 129/Sv x C57BL/6 control mice show a DC subset pattern similar to 10-week-old C57BL/6 control mice (data not shown).

Surprisingly, the frequency of CD11c^{high} DCs in 2-week-old TRAF6$^{-/-}$ mice was strikingly lower (<0.2%) than that of control, age-matched littermates, such that a distinct CD11c^{high} population was indistinguishable (Figure 3 and Table 1). Despite similar total splenic cellularity, TRAF6$^{-/-}$ mice exhibited a 5.6-fold reduction in the total number of splenic DCs compared to age-matched littermates (Table 1). Furthermore, the CD4$^+$ DC population was almost completely absent in TRAF6$^{-/-}$ mice compared to age-matched littermates (31-fold reduction). Though the CD8α$^+$ and double-negative (DN) populations were clearly identifiable in the small number of CD11c^{high} cells present in TRAF6$^{-/-}$ mice, their numbers were significantly reduced (5.5-fold reduction of CD8α$^+$ DCs and 3.3-fold reduction of DN DCs). The plasmacytoid DC population (CD11c^{lo}, CD45R$^+$, CD19$^-$, MHC class II^{lo}) was observed with the same frequency in the spleens of both TRAF6$^{-/-}$ and

Table 1. Cellularity of Splenic DCs

Mouse		Total Cells per Organ × 10^6	DC Numbers per Organ × 10^3 Total	$CD4^+$ DC	(%)[a]	$CD8\alpha^+$ DCs	(%)[a]	DN DCs	(%)[a]
C57BL/6	10-week-old	61.3 ± 7.0	957 ± 93	397 ± 57	(41.3 ± 2.5)	257 ± 25	(27.0 ± 3.5)	280 ± 38	(29.2 ± 1.5)
TRAF6 WT	2-week-old	41.7 ± 4.5	344 ± 56	106 ± 34	(30.3 ± 5.3)	94 ± 21	(28.3 ± 11)	132 ± 46	(37.4 ± 7.6)
TRAF6 KO	2-week-old	39.6 ± 3.4	61 ± 25	3.4 ± 1.9	(5.5 ± 2.1)	17 ± 5.5	(32.6 ± 18)	40 ± 25	(61.3 ± 16)
BM Chimera	TRAF6 WT	78.4 ± 17.6	1450 ± 319	935 ± 192	(64.8 ± 4.7)	167 ± 54	(11.3 ± 1.7)	312 ± 99	(21.4 ± 4.1)
BM Chimera	TRAF6 KO	46.6 ± 12.7	423 ± 121	38 ± 20	(8.8 ± 3.0)	200 ± 53	(47.9 ± 6.5)	172 ± 57	(40.3 ± 3.3)
RelB WT	7-week-old	56.7 ± 5.0	1030 ± 162	512 ± 67	(49.9 ± 1.3)	228 ± 70	(21.8 ± 3.3)	232 ± 36	(22.6 ± 1.3)
RelB KO	7-week-old	157.8 ± 11.1	1000 ± 324	131 ± 36	(13.2 ± 0.9)	411 ± 136	(40.9 ± 0.4)	426 ± 138	(42.4 ± 0.7)

Data represent means ± SD. At least three mice were used for each condition.
[a] Percentage of each subset in total DCs. Abbreviations: WT, wild-type; KO, knockout.

control mice (data not shown), indicating selective effects of TRAF6 deficiency.

TRAF6 Is Essential for the Development of $CD4^+$, but Not $CD8\alpha^+$, DCs

To determine whether the defect in splenic DC development in $TRAF6^{-/-}$ mice was a direct effect of TRAF6 deficiency in the hematopoietic compartment, as opposed to a secondary effect of growth retardation or the lack of a bone marrow cavity, we generated BM chimeras reconstituted with fetal liver cells from $TRAF6^{-/-}$ or wild-type littermate fetuses (designated $TRAF6^{-/-}$ BM chimera and wild-type BM chimera, respectively). Seven to twelve weeks after adoptive transfer of fetal liver cells, splenic DC populations were examined as described above. A distinct population of $CD11c^{high}$ cells (1.95 ± 0.16%, >99% of $CD45.2^+$ cells) was observed in the spleens of BM chimeric mice reconstituted with wild-type fetal liver cells (Figure 4 and Table 1). Analysis of DC subsets in wild-type BM chimeras revealed that the $CD4^+$ DC subset was predominant (64.8 ± 4.7%). In contrast, $TRAF6^{-/-}$ BM chimeras exhibited a markedly reduced frequency and total number of $CD11c^{high}$ cells (0.92 ± 0.21%, 3.4-fold reduction), in conjunction with a near complete absence of $CD4^+$ DCs (25-fold reduction). At the same time, $TRAF6^{-/-}$ BM chimeric spleens contained about half the number of DN DCs and a comparable number of $CD8\alpha^+$ DCs in comparison to wild-type BM chimeric spleens (Table 1). Taken together, these results indicate that TRAF6 regulates splenic $CD4^+$ DC development in vivo.

Defect in $CD4^+$ DC Development in RelB-Deficient Mouse

The NF-κB subunit RelB has been implicated in splenic $CD11c^+$ $CD8\alpha^-$ DC development but without distinction between the $CD4^+$ and DN subsets (Wu et al., 1998). To examine whether RelB, like TRAF6, regulates $CD4^+$ DC development, we analyzed splenic DC subsets in RelB-deficient mice. The frequency of $CD11c^{high}$ DCs was remarkably reduced in RelB-deficient mice compared to age-matched control littermates (more than a 2-fold reduction) (Figure 5 and Table 1). However, since RelB-deficient mice exhibit splenomegaly (nearly a 3-fold increase in total splenocytes), the total number of splenic DCs in RelB-deficient mice was found to be comparable to that of control littermates (Table 1). In comparison to control mice the frequency and absolute number of $CD4^+$ DCs in RelB-deficient mice were remarkably reduced (approximately a 4-fold reduction in each case) (Figure 5A and Table 1). Roughly twice as many $CD8\alpha^+$ DCs and DN DCs were found in RelB-deficient spleens as compared to control spleens (Figure 5A and Table 1). Thus, RelB seems to be required for $CD4^+$ $CD8\alpha^-$ DC development in vivo.

RelB forms potent transcriptional activators by associating with p50 or p52 (Ghosh and Karin, 2002), but the lack of p50 alone has been shown not to affect development of splenic DCs (Ouaaz et al., 2002). Thus, we examined splenic DC subsets in p52-deficient mice and found that they exhibit total $CD11c^{high}$ and $CD4^+$ DC populations similar in number and proportion to age-matched control littermates (Figure 5A).

Expression of TRAF6 and RelB mRNA in Splenic DC Subsets

To determine whether the differential effects of TRAF6 and RelB deficiency on splenic DC subsets correlate with their expression levels, we sorted each subset from wild-type spleens and prepared cDNA for mRNA analysis by real-time PCR. The normalized TRAF6 mRNA ex-

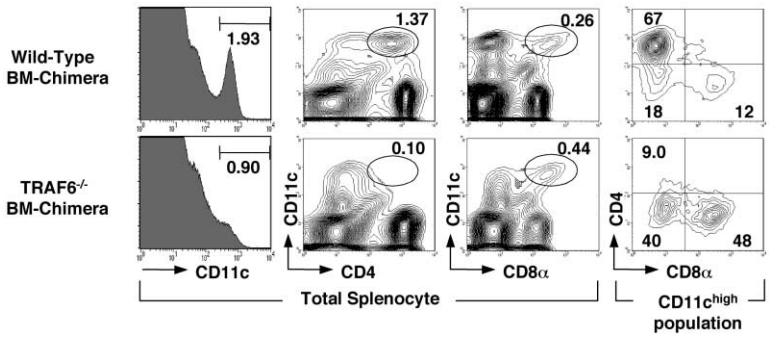

Figure 4. TRAF6 Is Essential for the Development of $CD4^+$, but Not $CD8\alpha^+$, DCs

Splenocytes from BM chimeric mice reconstituted with either $TRAF6^{-/-}$ or wild-type fetal liver cells were analyzed as described in Figure 3. Profiles of CD11c expression (far left panels), CD11c versus CD4 (middle left panels), and CD11c versus CD8α (middle right panels) in total splenocytes, and profiles of CD4 versus CD8α in $CD11c^{high}$ DC populations (far right panels) are shown. The data are representative of at least five mice for each condition.

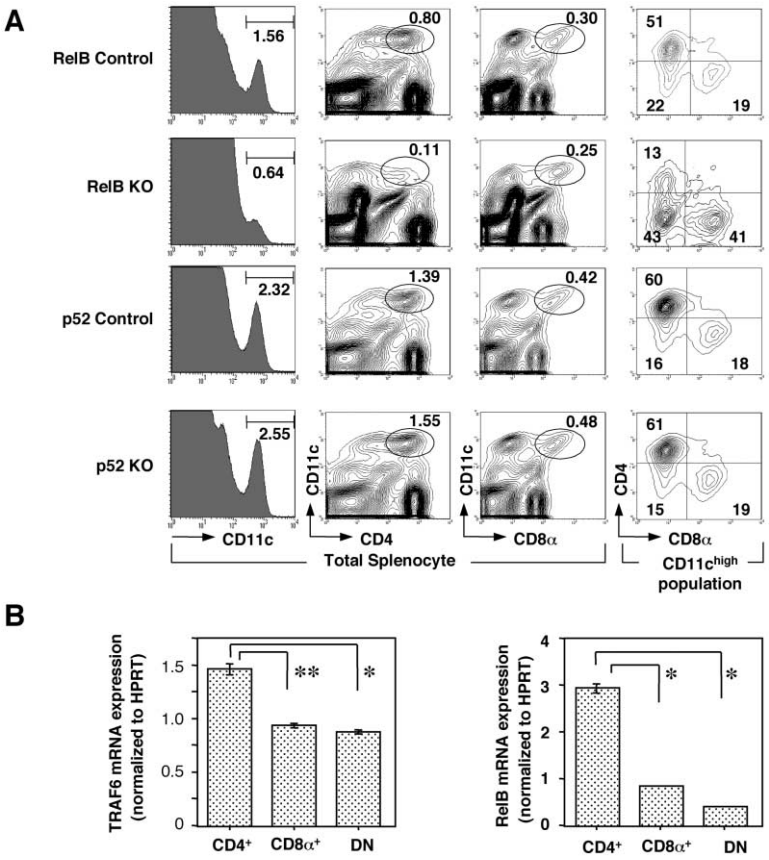

Figure 5. RelB Is Required for CD4$^+$ DC Development; TRAF6 and RelB mRNA Expression in DC Subsets

(A) Profiles of splenic DC subsets in RelB KO mice, p52 KO mice, and age-matched control mice are shown. The data are representative of at least four mice for each condition.

(B) CD4$^+$ DCs, CD8α^+ DCs, and double-negative (DN) DCs from wild-type spleens were sorted using a MoFlo cell sorter. RelB and TRAF6 mRNA expression levels in each subset were assessed by real-time PCR. TRAF6 and RelB expression levels were normalized to internal HPRT expression. The data indicate means ± SD of triplicate samples of one representative experiment out of three independent experiments (*, $p < 0.01$; **, $p < 0.05$).

pression level was approximately 1.5-fold greater in the CD4$^+$ DC subset than the CD8α^+ and DN subsets (Figure 5B). Also, the CD4$^+$ DC subset expressed roughly 3- or 6-fold more RelB mRNA than the CD8α^+ or DN subsets, respectively (Figure 5B). These results are consistent with the observation that defects in TRAF6 or RelB have the most profound effects on the CD4$^+$ DCs.

Discussion

TRAF6 and DC Maturation/Activation

The innate immune system triggers a host defense response against invading microorganisms via signaling by DCs through TLRs (Janeway and Medzhitov, 2002). Signals from TNFR superfamily members (e.g., CD40, TRANCE-R) also contribute to regulation of multiple functions of DCs (Banchereau and Steinman, 1998; Josien et al., 2000; Miga et al., 2001; Wong et al., 1997).

Although distinctions between DC maturation and DC activation remain unclear, previous reports have suggested that these processes are genetically distinguishable with respect to stimulation via microbial products or CD40L (Kaisho et al., 2001; Ouaaz et al., 2002). Our findings are of particular importance to understanding both DC "maturation" and "activation," which we define here, respectively, as (1) the upregulation of surface expression of MHC and other costimulatory molecules capable of enhancing proliferation of naive T cells, and (2) the production of inflammatory cytokines (e.g., IL-12) which regulate T cell differentiation. Though these definitions should not be accepted as absolute, they reflect current views in the field of DC biology (Banchereau et al., 2000; Banchereau and Steinman, 1998; Shortman and Liu, 2002). With that in mind, the data we have presented in this study regarding the role of TRAF6 in DC function should be addressed in the context of the ability of distinct immune receptor families, TLR and TNFR, to induce identical cellular responses (Figure 6).

DC Maturation

Microbial products have clearly been shown to stimulate DCs via distinct TLRs. For example, LPS- and CpG-DNA-induced DC maturation is abolished in TLR4- and TLR9-deficient mice, respectively (Hemmi et al., 2000; Kaisho et al., 2001; Kaisho and Akira, 2001).

However, despite the establishment of specific ligand-receptor pairs, it has remained unclear how such diverse microbial products and their cognate receptors could lead to a common DC maturation process. The simplest hypothesis suggests that a common intracellular signaling molecule used by all members of the TLR family, such as MyD88, mediates DC maturation. However, studies of MyD88$^{-/-}$ DCs have shown that while MyD88 is required for DC maturation by many of the TLR family members (e.g., TLR9), LPS-stimulated MyD88$^{-/-}$ DCs express normal levels of MHCII and costimulatory molecules, and efficiently induce T cell proliferation (Kaisho et al., 2001; Schnare et al., 2001). The results thus imply that, for DC maturation, there must be a MyD88-independent pathway utilized by some mem-

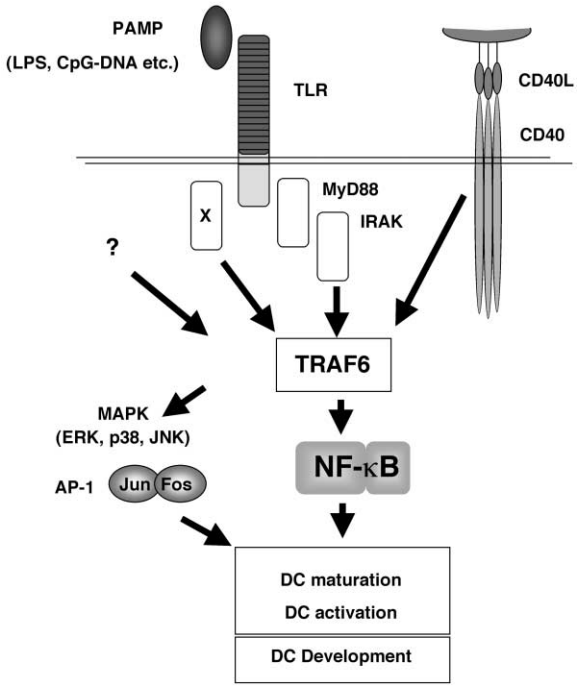

Figure 6. A Model for TRAF6-Mediated Signals which Regulate DC Functions

Signaling pathways mediated by TRAF6 regulate DC maturation and activation, as well as DC development. Upon recognition of PAMPs, TLRs recruit MyD88, which induces interaction between MyD88 and IRAK, allowing IRAK to form a complex with TRAF6. In addition, a pathway(s), which may involve additional adaptor(s) (designated as "X"), is engaged by TLR4 in response to LPS, resulting in DC maturation in a MyD88-independent manner. The TNF superfamily member CD40 associates directly with TRAF6. Signals from various TLRs and CD40 merge at TRAF6 and lead to subsequent activation of AP-1 and NF-κB. Together, these downstream signals and transcription factors regulate DC maturation and cytokine production. An unidentified ligand-receptor pair(s) (designated "?") regulates splenic DC development via a TRAF6-mediated process.

bers of the TLR family, including TLR4. Another adaptor protein in the TLR signaling pathway, TIRAP (also known as Mal), has been identified and shown to function downstream of TLR4, and thus may be partially responsible for the MyD88-independent response (Fitzgerald et al., 2001; Horng et al., 2001). However, LPS can still induce maturation in DCs from MyD88 and TIRAP double knockout mice, indicating that TIRAP is likely not critical to LPS-induced MyD88-independent DC maturation (Yamamoto et al., 2002). Therefore, to date there has existed little data supporting a model for convergence of DC maturation signals emanating from the TLR family gamut.

Our data shows that TRAF6, an adaptor downstream of MyD88 (and possibly TIRAP) (Figure 6), is required for DC maturation induced by TLR family members which utilize either MyD88-dependent (e.g., TLR9) or MyD88-dependent and -independent (e.g., TLR4) mechanisms. Therefore, these results provide evidence that there is an intracellular molecule representing a point of convergence for all signaling cascades activated by various microbial products and their cognate TLRs during DC maturation.

Moreover, our data argue that TRAF6 is also responsible for DC maturation induced by the TNF superfamily member CD40L. Together, these results indicate that both the TLR family members and CD40, a critical DC-regulatory TNFR superfamily member, utilize a common signaling pathway controlled by TRAF6 for DC maturation. Although TRAF6 activates several kinases (e.g., MAPK, IKK, PI3K, AKT) and transcription factors (AP-1, NF-κB), the specific requirement for each during either TLR- or CD40L-induced DC maturation remains unclear and will be the focus of future studies. Finally, it is important to note that there is likely not an intrinsic requirement for functional TRAF6 during DC maturation, as, for example, serial replating of TRAF6$^{-/-}$ DCs in culture plates induces maturation similar to that of wild-type DCs (data not shown).

DC Activation

One of the hallmarks of DC activation is the production of inflammatory cytokines, such as IL-12, upon stimulation with microbial products or CD40L. Several important observations have been reported regarding potential convergence of the signaling cascades induced by the TLR family and CD40 during induction of DC activation (Kaisho et al., 2001; Ouaaz et al., 2002). First, studies using MyD88$^{-/-}$ DCs clearly showed that MyD88 is essential for IL-12 production by DCs in response to all TLR ligands tested, including LPS. Since CD40 does not utilize MyD88 as a signaling adaptor (Figure 6), CD40-induced cytokine production by MyD88$^{-/-}$ DCs is unaffected. Second, NF-κB transcription factor subunits p50 and cRel are critical to DC activation as demonstrated by the failure p50$^{-/-}$cRel$^{-/-}$ DCs to produce cytokines in response to LPS or CD40L (Ouaaz et al., 2002).

The data we present in this study show that TRAF6 is required for DC cytokine production induced by either the TLR family or CD40. Our findings together with those of other groups suggest that TRAF6 is positioned at the point of convergence of NF-κB-dependent DC-activating signals emanating from CD40 or the TLR family.

TRAF6 and Splenic DC Subsets

In this study we also showed that TRAF6 is required for normal splenic DC development. In particular, CD4$^+$ DCs, the major splenic DC subset, were found to be almost completely absent in TRAF6$^{-/-}$ mice and TRAF6$^{-/-}$ BM chimeras. These results indicate that TRAF6 is required for proper development of CD4$^+$ DCs, a subset that appears to uniquely regulate T cell function among splenic DCs (Legge et al., 2002; Moser and Murphy, 2000; Shortman and Liu, 2002). However, it remains unclear whether the observed absence of the CD4$^+$ DC subset is due to an inability of CD4$^+$ DC precursor cells to further differentiate, a selective survival defect in the CD4$^+$ DC subset or a combination of both. Though TRAF6 appears to be required to some extent for maintenance of the DN DC subset, the relative significance may be less than for the CD4$^+$ DC subset, while the reduced numbers of CD8α$^+$ DCs observed in 2-week-old TRAF6$^{-/-}$ mice is likely an indirect effect of TRAF6 deficiency.

Since NF-κB is one of the major downstream targets of TRAF6 signaling, and RelB has been previously impli-

cated in CD8α⁻ DC development (Wu et al., 1998), it was important to reexamine the splenic DC subsets in RelB-deficient mice to see whether the defect is restricted to the CD4⁺ subset. Our results indicated that RelB-deficient mice show a selective defect in the CD4⁺ DC (Figure 5). Our data is, however, in contrast to the previous report which showed a near complete absence of all CD8α⁻ DCs in RelB-deficient mice (Wu et al., 1998). It should be noted, however, that the RelB-deficient mouse used in the previous study was generated by the accidental insertion of a transgene (Burkly et al., 1995; Wu et al., 1998), while the RelB-deficient mouse used in this study was made by homologous recombination (Weih et al., 1995). The different methods employed in generating RelB deficiencies may account for the inconsistent phenotypes observed.

Together these data suggest that TRAF6 and RelB operate in the same signaling cascade, with RelB likely downstream of TRAF6, and both are required for regulation of CD4⁺ DC development. However, the extent of the defect in CD4⁺ DC development in TRAF6$^{-/-}$ mice is more severe than that found in RelB-deficient mice. Considering the diversity of TRAF6 downstream targets (Figure 6), it is also possible that TRAF6 regulates CD4⁺ DC development in a RelB-independent manner. To provide insights into potential mechanisms regulating CD4⁺ DC development through TRAF6 and RelB signals, we examined mRNA expression levels of TRAF6 and RelB in each subset. Interestingly, we showed that TRAF6 and RelB were expressed most abundantly, though not exclusively, in CD4⁺ DCs. Whether the level of TRAF6 or RelB expression is an important factor for CD4⁺ DC development requires further study. However, it has been shown that quantitative differences in expression of a signaling molecule can affect the development of specific cell types. For example, high expression of c-Src in myeloid precursors affects osteoclast, but not DC or macrophage, development (Roodman, 1999). In either case, further studies are required to determine whether TRAF6- and RelB-mediated pathways regulating the fate of the CD4⁺ DC population are linked.

Conclusion

This study demonstrates a critical role for TRAF6 in both DC maturation and activation in response to microbial products and CD40L, and therefore provides evidence that signals generated by the IL-1R/TLR family and the TNFR superfamily member CD40L converge. In addition, we have provided evidence that TRAF6 is important for the development of DC subsets. Hence, these results imply that extracellular factors involved in DC maturation/activation might also be important for DC subset development and maintenance.

Experimental Procedures

Mice

TRAF6 KO mice were generated by homologous recombination in ES cells using the Cre/*loxP* system. A 15 kb DNA fragment containing exon 7 of *TRAF6* was isolated from a genomic DNA library. A 2.0 kb fragment of the exon and flanking introns was ligated with an *hsv-tk* cassette, a 5.5 kb long homologous (LH) fragment of the 5′ intron, a neo cassette in the 5′ region, a STOP cassette (Invitrogen), an IRES-EYFP cassette (Clontech), and a 1.5 kb short homologous fragment of the 3′ intron at the 3′ region, respectively. A *loxP* site was placed (in the same orientation) between the LH and the neo, between the neo and the exon, and between the STOP and IRES-EYFP fragments. The mutation in ES cells was identified by PCR with primers, T6-Screen1 (5′-GCTGGGAACAGTTTAACTTATGC-3′) and T6-Screen3 (5′-GGCTCTATGGCTTCTGAGGC-3′), and by Southern analysis using a 3′-external probe. The exon in the knockin ES clones was deleted by pMC-Cre transfection in vitro. The deletion was identified by PCR with primers, IRES/Cre-Screen1 (5′-CTTCGGCCAGTAACGTTAGGG-3′) and T6LH/Cre-Screen1 (5′-GTAAAGACAGAGAGTAGGTAGCCTG-3′), and by Southern analysis. The mutant ES clones were injected into C57BL/6 blastocysts.

RelB-deficient mice, p52-deficient mice, and OTII TCR transgenic mice have been described (Barnden et al., 1998; Caamano et al., 1998; Weih et al., 1995).

For generation of bone marrow chimeras, adoptive transfer of TRAF6$^{-/-}$ fetal liver cells was performed as described (Zheng et al., 2001).

Reagents and Antibodies

LPS, PGN, and LTA were from Sigma. Poly (I:C) was from Amersham Pharmacia Biotech. Phosphorothioate-stabilized CpG-DNA was synthesized as described (Hemmi et al., 2000). Antibodies were from BD Pharmingen (CD3; 145-2C11, CD4; RM4-5, CD8α; 53-6.7, CD11b; M1/70, CD11c; HL3, CD16/32; 2.4G2, CD40; HM40-3, CD45.1; A20, CD45.2; 104, CD86; GL1, I-A^b; AF6-120.1, I-A^d; 39-10-8, Thy1.2; 53-2.1; IFNγ; R4-6A2, XMG1.2, IL-4; BVD6-24G2, 11B11, IL-6; MP5-20F3, MP5-32C11, IL-10; JES5-16E3, JES5-2A5). Biotinylated antibody staining was followed by streptavidin-PerCP Cy5.5 (BD Pharmingen).

In Vitro DC Culture

DCs were generated from spleens of 2-week-old TRAF6$^{-/-}$ mice or BM cells from chimeras as described (Inaba et al., 1992) with some modifications for spleen-derived DCs. In brief, erythrocytes, T cells, B cells, and MHC II-positive cells were depleted, and 6×10^6 cells in 1 ml of medium (RPMI-1640, 5% FCS, 10 mM HEPES, 5×10^{-5} M 2-mercaptoethanol, penicillin [100 IU/ml]/streptomycin [100 μg/ml]) supplemented with 3.3% J558L-conditioned medium were cultured. On day 8, loosely adherent cells were harvested for experiments.

For phenotypic analysis of DC maturation, day 7 DCs were stimulated with LPS (10 ng/ml), poly (I:C) (20 μg/ml), CpG-DNA (10 μM), PGN (10 μg/ml), LTA (1 μg/ml), or 1:200 dilutions of CD40L-containing supernatant (Wong et al., 1997) for 24 hr, harvested on day 8, and analyzed with anti-CD11c, anti-CD86, and anti-I-A^b Abs. For cytokine assays, cells were treated with stimuli for 48 hr, and supernatants harvested on day 8. IL-12 p40 levels were measured using mAb C17.8 and biotinylated mAb C15.6, and IL-6 levels using mAb MP5-20F3 and biotinylated mAb MP5-32C11, as described (Speirs et al., 2002). For biochemical studies, day 7 DCs were harvested, and CD11c⁺ CD45.1⁻ DCs sorted. Purified DCs were cultured at 2×10^6 cells/500 μl in serum-free RPMI at 37°C for 3 hr. DCs were stimulated with 100 ng/ml of LPS for the indicated times and subjected to Western and real-time PCR analyses.

In Vivo DC Analysis

For induction of DC maturation by LPS in vivo, 2-week-old TRAF6$^{-/-}$ mice and control littermates were injected i.p. with LPS (100 ng/g body weight) or PBS, and DCs assayed 6 hr later. For CD40-induced DC maturation, mice were injected i.p. with anti-CD40 Ab (FGK115; 50 μg/mouse) or PBS, and DCs examined 27 hr later. Single-cell suspensions were prepared from spleen and analyzed by flow cytometry.

Adoptive Transfer of Allogeneic DCs

DCs generated by in vitro culture of spleen cells, as described above, were stimulated with LPS or untreated. Cells were washed and injected (0.5×10^6 cells) i.v. into Balb/c mice. After 7 days, splenic T cells from naive or DC-primed Balb/c mice (haplotype d) were enriched, labeled with CFSE (Molecular Probes Inc., Eugene, OR) as described (Wells et al., 1997), and used as responders. 2×10^5 total cells were placed in 96-well plates, and varying numbers of

γ-irradiated (2000 rads) T cell-depleted splenocytes from TRAF6 heterozygous or wild-type mice were added as stimulator cells (haplotype b). After 72 hr, cells were harvested, stained with anti-CD3 antibody, and analyzed by flow cytometry. Mitotic index was determined as described (Wells et al., 1997). IFN-γ, IL-4, and IL-10 were measured by ELISA.

Adoptive Transfer of Antigen-Loaded DCs

Bone marrow from chimeric mice was cultured for 8 days with GM-CSF. From days 4–8 medium was supplemented with 100 μg/ml of whole chicken OVA protein (Worthington Biochemical Corporation). Cells were replated on days 6 and 7, and medium left untreated or treated with 10 ng/ml LPS from days 7 to 8. 1.5×10^5 BMDCs were resuspended in PBS and injected into two CD45.1 congenic male recipient mice. Additional recipient mice were injected with PBS alone. Twenty-four hours prior, all CD45.1 recipient mice had been injected i.v. with 1.4×10^6 purified CFSE-labeled OTII $CD4^+$ T cells (CD45.2). Four and a half days after immunization, spleens and lymph nodes were harvested, and donor T cells identified with antibodies specific for CD45.2, CD4, and Thy1.2.

Real-Time PCR

TRAF6, RelB, and IFN-inducible gene mRNA expression was assessed using the primers below in combination with SyBr Green (Applied Biosystems). PCR primers: TRAF6 sense (5′-AACTGTGCT GTGTCCATGGC-3′) and TRAF6 antisense (5′-CAGTCTCATGTG CAACTGGG-3′) for TRAF6; RelB sense (5′-AGCAGCTCTGATCCA CATGG-3′) and RelB antisense (5′-AGCACTGGCAAGTGGTGGTG-3′) for RelB; GARG16 sense (5′-AGGCAGGACAATGTGCAAGAA-3′) and GARG16 antisense (5′-CCATGCAAACATAGGCCATCT-3′) for GARG16; IP10 sense (5′-TCTGAATCCGGAATCTAAGACCAT-3′) and IP10 antisense (5′-GGTGTGTGCGTGGCTTCA-3′) for IP10; IRG1 sense (5′-ATTCGGAGGAGCAAGAGGATGAT-3′) and IRG1 antisense (5′-GCTGGAGGTGTTGGAACTGTAGAT-3′) for IRG1; 5′-hypoxanthine ribosyltransferase (HPRT) (5′-GTAATGATCAGTCAACGG GGGAC-3′) and 3′-HPRT (5′-CCAGCAAGCTTGCAACCTTAACCA-3′) for HPRT. Detection of HPRT was used as an internal control. The PCR program was as follows: 50°C, 2 min; then 40 cycles (95°C, 1 min; 60°C, 1 min). Signal detection was performed using an Applied Biosystems 7700 Sequence Detector (Sequence Detector v1.7a software).

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